STUDIES ON PRODUCTION OF ANTIBIOTICS BY INDUCTION OF MARINE BACTERIA WITH HUMAN PATHOGENS

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT

FOR THE DEGREE OF

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By

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on production of antibiotics by induction of marine bacteria with human pathogens"** which is being submitted by **Ms. Rejitha Nair**, Roll No. 410LS2062, for the award of the degree of Master of Science in LIFE SCIENCE from National Institute of Technology, Rourkela, is a record of bonafied research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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DECLARATION

I Rejitha Nair, M.Sc. Life Science, Department of Life Science, N.I.T., Rourkela hereby declare that my research work incorporated in the dissertation titled “Studies on Production of Antibiotics by Induction of Marine bacteria with Human Pathogens” is an authentic research work carried at Department of Life science, National Institute Technology, Rourkela under the direct guidance and supervision of Dr. Surajit Das, Asst. Professor, Department of Life science, NIT, Rourkela. The project work is original and no part of this work has been submitted for any other degree or diploma. All the given information is true to best of my knowledge.

Rejitha Nair

Date:

Place:

ACKNOWLEDGEMENT

This project is by far the most significant accomplishment in my life and it would not have been possible without the people who supported me and believed in my caliber.

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ABSTRACT

Cross species signal transfer mediated induction of antibiotic production by the marine bacteria against common bacterial pathogens was investigated in the present study. Marine bacteria were isolated and analyzed for their efficacy in antibiotic production against common clinical pathogens viz., *Pseudomonas sp., Escherichia coli, Bacillus subtilis* and *Proteus* sp. Out of 36 bacterial isolates analyzed, four isolates exhibited significant antibacterial activity against *Pseudomonas, Bacillus, Proteus, Klebsiella and Escherichia coli* Isolate designated as CW 602 produced antibiotic when co-cultured with *Pseudomonas* and CW 401 produced antibiotic when co-cultured with *Bacillus* cells. Cell free extract of CW 602 and CW 401 cultivated in the presence of heat killed *Pseudomonas, Bacillus* cells was subjected to solvent extraction by ethyl acetate and antimicrobial activity was tested by disc diffusion method. Present study on induced antibiotic production by *Pseudomonas, Bacillus* in bacteria CW 602 and CW 401 pave the way for the discovery of pathogen targeted/ specific antibiotic production.

Keywords: Cross Species Signal, Anti bacterial activity, Co- culture, marine bacteria

1. INTRODUCTION

1.1 Antibiotics and Diseases

Antibiotics are medicines that kills bacteria or slows the growth of bacteria. They are used to cure diseases. Antibiotics were first produced in 1939. The term antibiotics was introduced by S.A Waksman in 1942. Antibiotic is very different from chemotherapeutic drugs, antibiotics are natural drugs that is produced by several fungi or bacteria but chemotherapeutics drugs are man-made substances. History of antibiotics began in 1932, (Nussbaum., et al,2006) when the first drug sulfonamide was prepared. Sulfonamides are effective drugs. Sulfonamides have shown tremendous positive results on urinary tract infections, *shigellosis and Pneumococcal pneumonia*. Penicillin was active on *pneumococci* and *streptococci*. They have inhibitory effects on *Cornybacterium diphtheria* and *Treponema palladium.* Streptomycin were effective on Gram negative aerobic bacteria and *Mycobacterium tuberculosis*. The problems of infectious diseases suddenly increased when certain became antibiotic resistant (Walsh., 2003). *Mycoplasma, Clamydiae* or *Rickettsiae* were not affected by penicillin or streptomycin. Staphylococci species were the first pathogens to show resistance and their resistance and their resistance spread throughout the world in a short period of time. This problem was solved when new antibiotics such as neomycin and colimycin were discovered. Semi- synthetic antibiotics like methicillin and amphicillin were prepared from penicillin molecule. These drugs were effective against staphylococcus species and gram- negative bacteria like *E coli, H. influenza*. This marked the production of semi- synthetic drugs in large scale. Pathogens such as *pseudomonas, serratia. Acinetobacteria* found in hospital environment showed inherited resistance to antibiotics (Raju et al., 2000). To overcome this modern aminoglycosides, anti-pseudomonadal penicillins and other beta lactums were produced. Antibiotics such as teicoplanin, triazol, antimycotics were discovered to treat patients with weak immune system. Oral cephalosporins, new macrolides deoxycyclin and fluroquinol were used for the treatment of community infections. Beta lactam are the bacterial drugs that inhibit the bacterial cell wall by inhibiting the synthesis of peptidoglycan. Bacterial enzymes that are very much affected by the action of beta lactam are called “Penicillin Binding protein”. Half life of beta lactams such as oxacillin, cefopergon and

cephalotin is 2-5 hours. Penicillin are active against streptococci, pneumococci and most anaerobes like clostrial species. Cephalosporins such as cefalotin and cefazolin are used for the treatment of skin and soft tissue infections. Cefaclor are active against ***haemophilus*** species. Cefuroxin and ceftriaxon are used for the treatment of pathogens like ***E. coli, H. influenza, meningococci, salmonellae.*** Cefazidin is the most strongest anti- pseudomonadal cephalosporin. Aztreonam is active against ***enterobacteriaceae, pseudomonas*** and other gram negative aerobic organisms. High dosage of vancomycin causes flushing release of histamine leading to eosinophilia (Hugenholtz et al., 1997). Vancomycin is given orally to the pathogenic bacteria located in the intestine, for example Colitis caused by *Clostridium difficile*. Teicoplanin has high penetrating power in the tissues. Streptomycin, used for the treatment of tuberculosis. Tohramycin is effective against *pseudomonas*. Erythromycin used for curing hepatic damage. Co-trimoxzol effective against *pneumococci, styphalococcia* and *E coli* (Amann et al., 1990).

1. 2 Methicillin Resistant *Staphylococcus aureus*(MRSA)

MRSA (Methicillin Resistant *Staphylococcus aureus*), they are the strains that has developed resistance to beta- lactam antibiotics. Strains which are not resistant to these antibiotics are classified as Methicillin- sensitive *Staphylococcus aureus* (Daum and Robert., 2007). *S aureus* are commonly present in the regions like nostrils, open wound. Patients having compromised immune system are at a greater risk of infection. MRSA can be detected by swabbing the nostrils and isolating the bacteria (Chang et al., 2003). Lesions developed due to MRSA are initially small pimple like structures that turn into pus filled boils in the later stages. It can lead to the damage of vital organs and necrotizing pneumonia (“flesh eating”). Many MRSA are deadly.

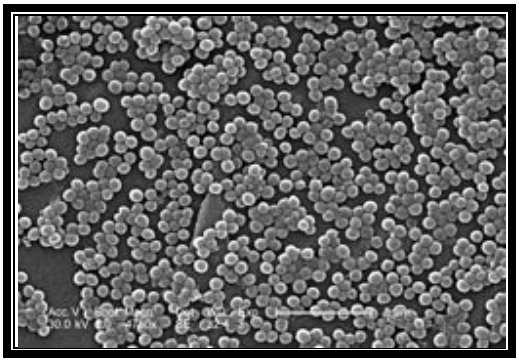

Fig. 1: Scanning electron microscopic view of MRSA

1.3 Vancomycin Resistant *Staphylococcus aureus* (VRSA)

VRSA (Vancomycin Resistant *Staphylococcus aureus*), they are the strains that have become resistant to glycopeptides antibiotic vancomycin. High level vancomycin resistance has been seen in *S.aureus*(Ena et al., 1993).

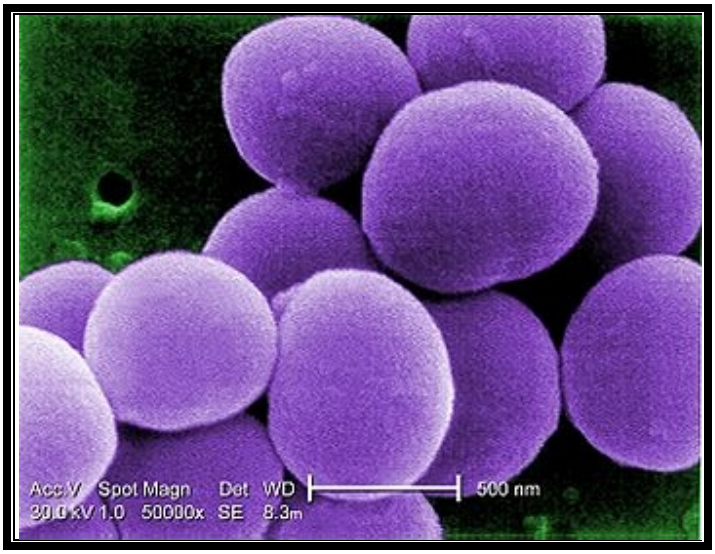

Fig.2 : Scanning electron microscopic view of VRSA

Low level vancomycin resistance in coagulase negative ***staphylococci*** has been reported. The first VRSA has been reported in Japan. The names were MU 50 and MU 3 respectively.

75% of the earth's surface is covered with marine organisms. Thus the future of this marine technology is quite promising. Marine organisms turn out to be a source of anti- cancer compound for example Didemin, which is isolated from *Carribean tunicle*. *Trideodemnum solidum* acts against leukemia and melanoma. Most of the marine bacteria come under gram negative category. Marine bacteria are richly endowed with enzymes. *Thermococcus litoralis,* archaebacteria produces viral DNA polymerases that can remain active for more than 2 hours at 100'C. Thus this shows that marine bacteria have the potential to synthesize self tolerant enzymes that can be used in food and pharmaceutical industries, as such they are used for therapeutic purposes.

1.4 Quorum Sensing and Cross Talk

Earlier bacteria was considered to be an individual cell that itself had the capability to multiply in presence of nutrients. But recently, intercellular communication in bacteria has been discovered, which was once believed to be only in multicellular organisms. The advantage of this mechanism is that they will become more adapted to sessile environment, will have better nutrient supply and new modes of growth. This phenomenon of cell to cell communication is called Quorum Sensing.This intercellular communication is based on the small self-generated molecules called autoinducers. With the help of these autoinducers bacteria can regulate their behavior accordingly with the population density. The main principle of Quorum Sensing is when a single bacteria releases autoinducers into the environment, their concentration is too low to be detected but in presence of another bacteria the autoinducer reach to a threshold level and allows the bacteria to sense a critical cell mass and in response activate or repress target genes.

2. REVIEW OF LITERATURE

The serendipitous observation of penicillin antibiotics marked the beginning of the modern era of antibiotic discovery by Alexander Flemming in 1928 (Alexander., 1928). According to him when *Penicillin notatum* were grown in appropriate substrate it would extrude a substance that has antibiotic properties. John Tyndall of Royal Society in 1875 first published this work in his book " Elements of General and Medical Botany" (Flemming., 1928). Viceyo Tiberio in 1895 who was a physician in the University of Naples published a research, regarding the mould in well that contained some anti-bacterial activity (website). Picado Twight in 1915 observed inhibitory actions of fungi genus penicillium.In 1871 Joseph lister experimented on penicillin for aseptic surgery as it weakened the growing microbes. Flemming also observed the growth of staphylococcus which he had left open and it was further contaminated by blue-green mould, which actually formed a visible growth around the mould. He arrived at a conclusion that the moulds arrived at a conclusion that the moulds released a substance that inhibited the growth of the pathogen. Thus penicillin was effective only against Gram positive bacteria and ineffective against gram negative bacteria. It also used for the isolation of *Bacillus inflenzae.* In 1930, Cecil George Paine recorded the first disease to be cured by penicillin (Wainwright et al., 2001). The term antibiotic was introduced by S. A Waksman in 1942. In 2001 Walve concluded that the order *Actinomycetales* had yielded 300 antibiotics so far. (Walve et al., 2001). In 2005, Baltz noted that out of 2500 strains of antibiotics, 250 would make streptothricn, 125 could make streptomycin and 40 would make tetracyclines with frequencies of 2x10, 1x10 and 4x10 respectively (Baltz and Marcel., 2006). In 1976, Arial observed the frequencies of vancomycin and erythromycin and were found to be high (Arial., 1976). Based on a study in 2001-2002, it was observed that an epoxy lactone with methylated olifinic side isolated from the *Aspergillus niger* culture excreted antimicrobial activity against *Haemonchus contortus* (Omura et al., 2001). The marine natural product omnafide F, which was isolated from the Australian marine sponge *Trachycladus laevis* pinulifer showed potent inhibitor of larval development of the parasite nematode *Haemonchous contrortus* (Vuong et al., 2001). In 2002, Torres and co-workers investigated the arenooclerins A and haliclonacyclanine E, which are the tetracyclic alkylpiperidine alkaloids isolated from marine sponge Arenosclera brasiliensis. According to the

investigation the bio-piperdine ring had played a significant role in the antibiotic activity of these compounds against antibiotic resistant *Staphylococcus aureus*. These compound further form the advanced drugs. Linington in 2002 developed an assay to screen marine compounds which inhibited a secretory item in *E coli*. This has resulted in the isolation of anti-microbial glycolipid caminoside A from the marine sponge Caminus spaeroconia. Caminoside A was potent again methicillin resistant *S. aureus* and vancomycin resistant enterococcal strains(Linington et al., 2002). The isolation of peptide bogorol A from a marine bacillus culture was active against methicillin resistant *S.aureus* (Barsby et al., 2002) and vancomycin resistant enterococcal strains. An unusual peptide was discovered dicynthaurin was discovered from haemocytes of marine funicate *Haalocynthia aurantium* (Lee et al., 2001). Halocin was discovered from H. aurantium (Jang et al., 2002). This halocidin demonstrated significant potency against mathicillin- resistant *S.aureus* and multidrug- resistant *Pseudomonas aeruginosa*. Glycoside, iyengaroside was isolated from marine green algae *Codium iyengaric* (Ali et al., 2002) showed less potency against gram negative and gram positive bacteria as compared to tetracycline. Vairappan repoted G15 acetogenin, lembyne-A from marine red alga *Laurencia* species (Vairappan et al., 2001). Two novel halogenated sesquiterpenoids, pannaosanol and pannosane from red alga, *Laurencia pamnosa* (Suzuki et al., 2001). Pestalone showed potent antibiotic activity against methicillin-resistant *S- aureus* and vancomycin resistant *Enterococcus faecium*. Isolation of furan carboxylic acid from *Cladosporium herbarium,*a marine fungus was found to be active against *Bacillus subtilis* and *S. aureus* (Tian et al.,2001). Zamastatin was derived from okinawan sponge *Pseudoaratina purpurea* (Takada et al., 2000) and zamastatin affected the growth of marine biofouling bacteria *Rhodospirillum salenigens*.

Staphylococcus aureus was recognized as a major reason of hospital acquired infections and they have become endemic. In 1980 because of the widespread occurrence of MRSA, empirical therapy for staphylococcal infections changed to vancomycin in health care institutions. In 2001 massive rise in skin and soft tissue was reported in Los Angeles Country Jail System. By 2002 it wa srepoted that S. aureus accounted for about 70% . Lowry and his colleagues reported the demonstrated frequent colonization with MRSA. Hundreds of MRSA outbreaks appeared between 2000 to 2008. In February 2008, Tulsa Country Jail in U. S State of Oklahoma starting treating an average of about twelve *staphylococcus* cases per month (Graveland et al., 2011). Based on a study in 2011, 47% of the meat and poultry sold in U.S grocery stores was

contaminated with *S. aureus* and 52% were resistant to antibiotics. On 6th January 2008, half of the cases of MRSA infections in Hong Kong were domestic helpers (Graham et al., 2006). In June 2002 new strains of MRSA Clonal complex 398 was responsible for Livestock- associated MRSA infections (Hidron et al., 2008). Studies published on 2004-2007 reported that hydrogen peroxide vapor could be used as a decontaminant in hospital rooms. According to the European countries Anti- microbial Resistance Surveillance Network 2008 data, there was a significant declining trend of invasive MRSA infections observed in Austria, Latria, France, Romania, Poland, Italy, UK, Belgium over the last four years. There was a significant decrease in MRSA in ICU- acquired MRSA infections between 2004-2007 (Europe., 2010).Thus from the data it has been concluded that the incidence of MRSA infections has been reduced in european countries.

During the early 1990 there was a discrenable increase in vancomycin use as a result which had lead to the emergence of S.aureus strains and other species of staphylococci with decreased susceptibility to vancomycin and other glycopeptides. In 1997 the first strain of Staphylococcus aureus which had reduced susceptibility to teicoplanin and vancomycin was reported from Japan.

Quorum Sensing depends on population density (Hiramatsu et al., 1997). On the other hand extracellular signaling forms a basis for the control of molecules and cellular process and population behavior. Quorum sensing is of two types, firstly species specific and secondly, interspecies. Species specific is more common in Gram negative bacteria which is mediated by acyl homoserine lactones(AHL)(Fuqua et al., 2001). Species recently discovered with interspecies communications has been linked to autoinducer-2(AI2), which is a furanosyl borate diester (Chen x et al., 2002). In *E coli*, a recent review of cell to cell signaling has a concise description of AL-2 signaling (Ahmer., 2004). Quorum sensing regulates competence development, antibiotic synthesis, cell differentiation, sporulation, virulence factor induction and nutrient flux (Cvitkovitch et al., 2003). Recently quorum sensing was linked through proteomic analysis in order to increase pathogenic competence in strains of *Pseudomonas aeruginosa* (Arrevalo et al., 2004). Recent research studies have shown that acyl homoserine lactone (AHC) which are signals from *Pseudomonas aeruginosa* can enter mammalian cells and can also activate artificial transcription factors (Williams et al., 2004). Thus we can say that although few researches have been done in this field but still it is interpreted that cell induced antibiotic

production generates a high rate of antibody as compared to bacteria solely. Research is still going on.

This coordinate behavior of cell-density-dependent has several advantages. In pathogenic microorganisms, the regulation of the virulence determinants throughout the infection process plays an important role in pathogenicity.The major goal of pathogens are to evade disease, and as such, quorum sensing is an important asset because it enables bacteria to appropriately time expression of immune response-activating products. Using quorum sensing, bacteria can amass a high cell density before virulence determinants are expressed, and the bacteria are able to make a concerted attack and produce virulence factors to overwhelm the host defenses.

3. OBJECTIVES

Importance of this study is to compare the rate of antibiotic production by the bacteria solely and when it is induced with another bacteria Quorum Sensing forms the basis for cell induced antibiotic production.Bacterial cells have the ability to show cell to cell communication in presence of another bacteria with their autoinducers. This allows the bacteria to sense a critical cell mass and in response activate or repress target genes.

- Isolation and enumeration of from marine bacteria.
- Screening for antimicrobial activity of isolated marine bacteria
- Extraction of crude compound from the marine bacteria culture
- Study species signal transfer mediated induction of Antmicrobial compounds in Marine Bacteria.
- Characterization and identification of potent Marine Bacterial strains.

4. MATERIALS AND METHODS

4.1 Sample Collection

A study was done on 16 different isolates of marine bacteria, all water samples were kept at 4 ‘ C until transferred to the laboratory. The samples were collected from five different places. They are:- Bhitarkanika, Paradip, Chilika and Rushikulya respectively.

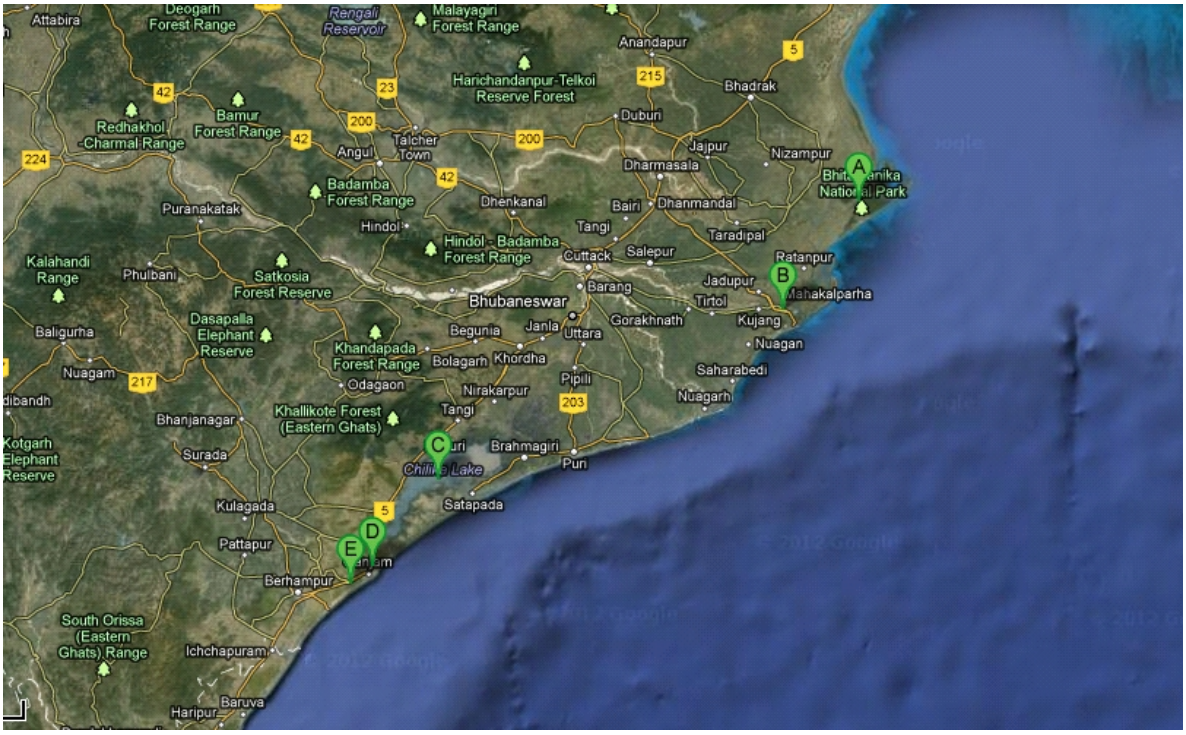

Fig.3: Sample Location(A- Bhitarkanika; B- Paradip; C- Chilika Lake; D- Rushikulya; E- Gopalpur)

4.2 Isolation of marine bacteria

1ml of the water sample was mixed with 9ml of distil water and serial dilution was performed. After this 0.5ml of the aliquots were spread on the surface of the ZMA(3 plates). The inoculated plates were incubated at 24, 30 and 37'C for 3-5 days. After incubation streaking is performed and we will select 4 isolates based on their pigmentation, size , elevation and margin. (Webb J S et al., 2003)

After the growth of the colonies on ZMA plates the strains were re streaked on Sea Water Nutrient Agar plates.

4.3 Screening for anti- microbial activity

4.3.1 Cross Streak Method:

The strains were cross streaked against the pathogens on MHA plates. The strains were streaked horizontally on the MHA and the 5 pathogens were streaked vertically towards the strain till a marginal line. The pathogen crossing the marginal line is taken as positive result. The results of the strains against the pathogens were represented in a tabular form.

4.3.2 Agar Well Diffusion Method:

5 test tubes were prepared each containing 2ml of LB. After autoclaving the pathogenic strains were inoculated in it and kept in shaker at 180 rpm at 37'C. The autoclaved ear buds were dipped in this broth and swabbed on the MHA plates. Borings was performed on MHA plates and bore wells were formed. The 4 test bacterial strains were inoculated in 5ml of NB after autoclaving. It was kept on shaker for 48 hours. The cultures of the strains were poured on to the wells. The cultures were then centrifuged at 6000 rpm for 10 minutes. Their supernatants were then poured in the wells. The plates were then kept for 48 hours incubation. The inhibition zones were observed.

4.3.3 Disc Diffusion method:

The selected strains were inoculated in 50 ml of Zorbell's Marine Broth and was kept for shaking in 160rpm at 32'C for 48 hours. After 48 hours 5 ml of broth culture was centrifuged at 5000 rpm for 10 minutes at 4'C. 1ml supernatant was collected and added with 1ml ethyl acetate. Then supernatant with ethyl acetate was shaken for 2 minutes and the crystal layer formed on the surface was pipetted out. It was then kept for evaporation up to 24 hours. After evaporation the crude extract was collected. The crude extract was then mixed with 2ml of DMSO and the extract was made to dissolve in it. Small disc was punched out from the filter paper and autoclaved. The 5 pathogenic strains were grown in LB. 100µl of the pathogenic strains were spread on the MHA plates. The filter discs were dipped in the crude extract solution mixed with DMSO and then kept on the MHA plates. The plates were then kept for 48 hours incubation. The zones were observed around the filter paper.

4.4 Enhancement of Anti-microbial Compound synthesis by cross cell induction:

25 ml of each bacterial strains cultures were made in falcon tubes such that 4 sets(for 4 strains) were prepared. Each set contained 3 tubes.

- 1st tube- Blank(no pathogens)+ Bacterial strains
- 2nd tube- heat killed pathogens+ Bacterial strains
- 3rd tube- Live pathogens+ Bacterial strains

Then it was kept for 3 days incubation and again the above disc diffusion steps were repeated.

4.5 Biochemical identification of bacterial strains

Biochemical identification of the selected strains was performed by physical and biochemical characterization.

4.5.1 Physical Characterization

a) Gram Staining: The diluted suspensions of bacteria were smeared on clean slides, air dried, heat fixed by passing over a flame for 2 to 3 times. The slides were flooded with crstal violet solution for one minute, washed with water and flooded with gram's iodine for one minute. The slides were washed with water and decolorized with 95% ethyl alcohol dropped from a dropping bottle until no violet colour was visible from drain off solution.The slides were washed with water and counter stained with safranin stain for about 30 second and washed with water. The slides were air dried and examined under a microscope using 100 X objective using a day light filter. Cells were then identified by the colour observed purple for Gram positive and pink for Gram negative cells.

4.5.2 Biochemical characterization

Hi Media Rapid Biochemical Bacillus Identification Kit was used. Kit was opened aseptically and sealing tape was peeled off. Each well was inoculated with 50µl of the above inoculums by surface inoculation at 35-37'C for 18- 24 hours. At the end of the incubation period, a series of reagents were added in designated wells as per manufacturer's specifications to carry out different biochemical tests.

5. RESULTS

5.1 Screening Of antibacterial Activity-

5.1.1 Cross Streak Method

This study was done by cross streak method against five pathogenic strains of marine bacteria namely E coli, Proteus, Klebsiella, Pseudomonas and Bacillus.

TABLE 1-, Cross Streaking of the strains against five pathogens(Ecoli, Proteus, Klebsiella, Pseudomonas and Bacillus respectively)

Strains	*E coli*	*Proteus*	*Klebsiella*	*Psuedomonas*	*Bacillus*
BW03	+	-	+	-	-
PW08	-	-	-	-	-
PW03	-	-	-	-	-
PW201	+	-	+	-	-
PW307	-	-	+	-	-
PW02	-	-	-	-	-
CW602	-	-	+	+	-
RW203	+	-	+	-	-
CW601	+	+	-	-	-
CW102	-	-	-	-	-
CW101	-	-	-	-	-
RW507	-	-	+	+	-
CW401	-	+	+	-	+
CW603	-	-	-	-	-
CW103	-	-	-	-	-
RW102	-	-	-	-	-

Among the above results *Klebsiella* has shown good results and Bacillus has shown least inhibition against the strains.

Fig.4: (a)Cross Streaking of CW 401 against pathogens

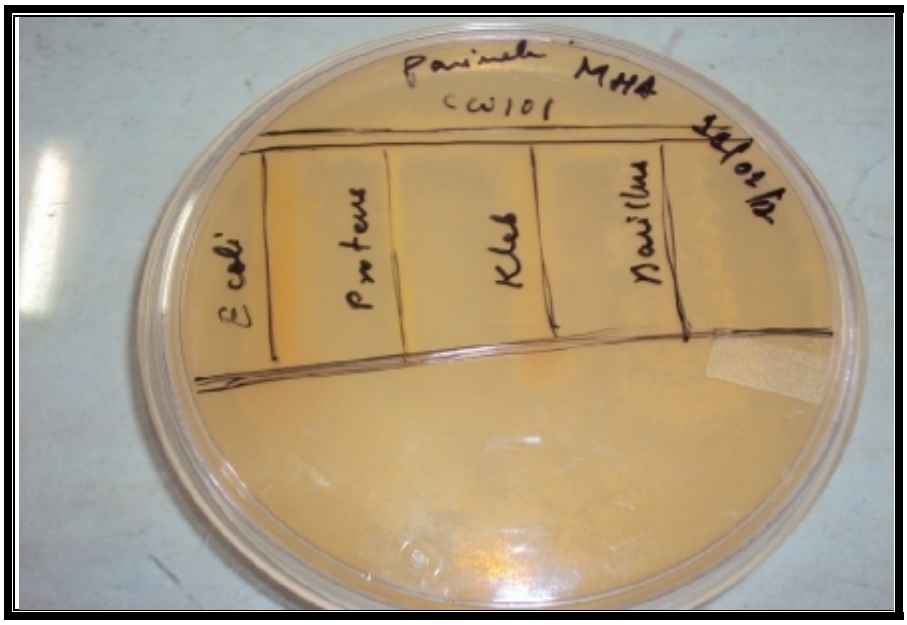

Fig. 4 : (b) Cross Streaking of CW 601 against pathogens

5.1.2 Agar Well Diffusion Method

These results are based on the screening of antimicrobial activity using the cultures of strains and their supernatants separately.

TABLE 2- Screening of antimicrobial activity by Agar Well Method

Strains	***E coli***	***Proteus***	***Klebsiella***	***Pseudomonas***	***Bacillus***
BW03	-	-	-	-	-
PW08	-	-	-	-	-
PW03	-	-	-	-	-
PW201	-	-	-	-	-
PW307	-	-	-	-	-
PW02	-	-	-	-	-
CW602	-	-	-	+	-
RW203	-	-	-	-	-
CW601	+	+	-	-	-
CW102	-	-	-	-	-
CW101	-	-	-	-	-
RW507	-	-	-	+	-
CW401	-	+	-	-	+
CW603	-	-	-	-	-
CW103	-	-	-	-	-
RW102	-	-	-	-	-

Fig .5 (a) : Screening of antibacterial activity by Agar well method

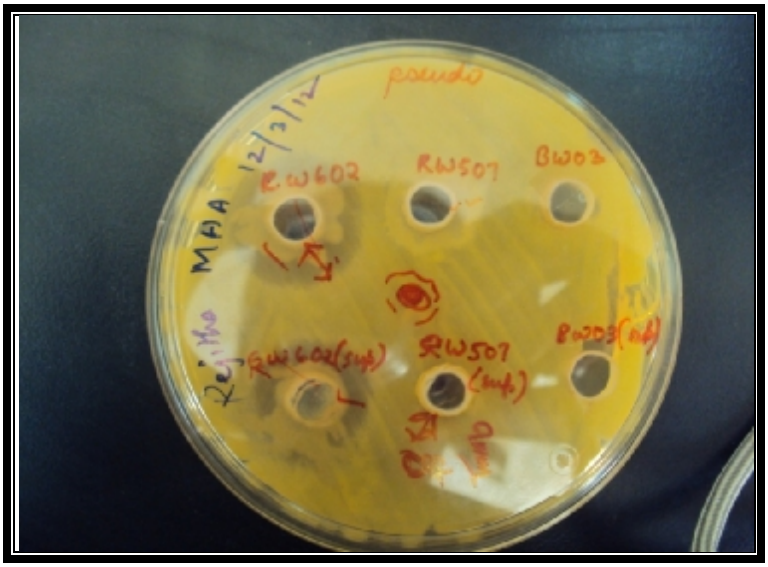

Fig .5 (b) : Screening of antibacterial activity by Agar well method

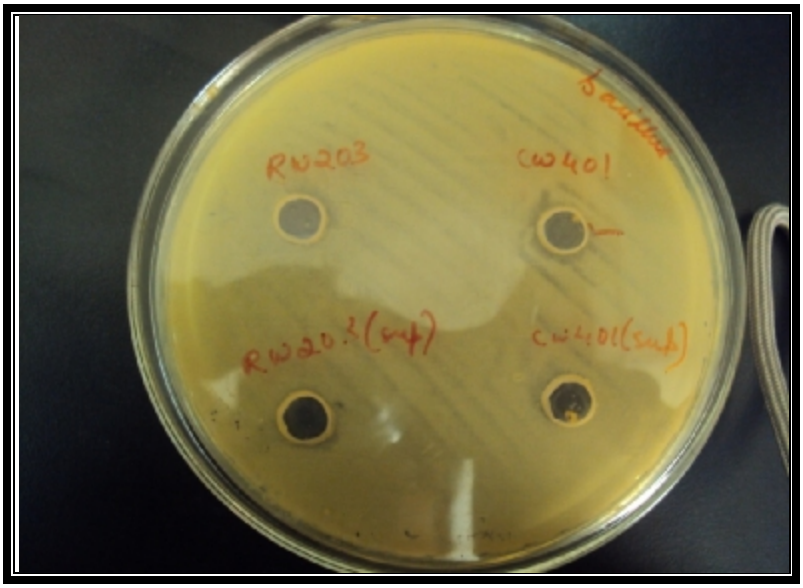

Fig .5 (c) : Screening of antibacterial activity by Agar well method

Finally selected strains

CW401, RW507, CW601, CW602

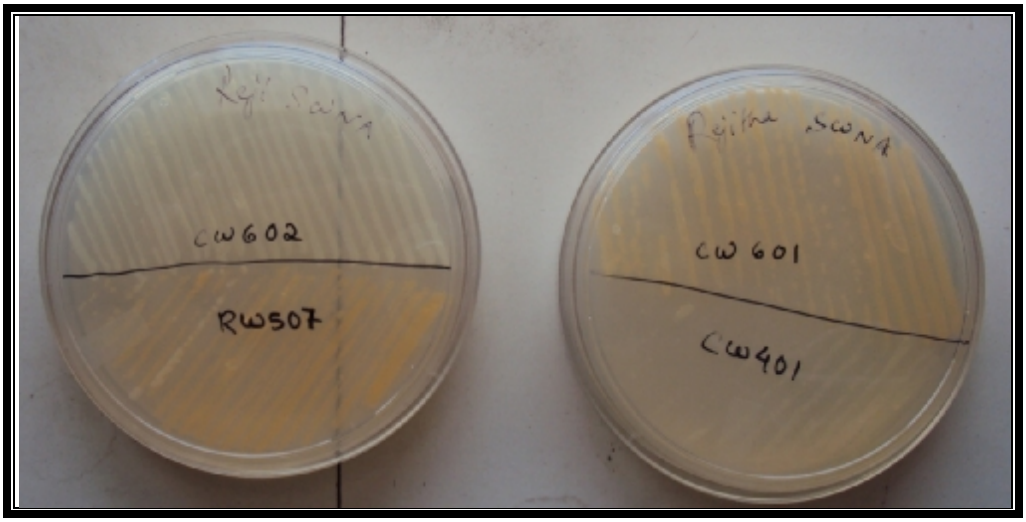

Fig. 6 : CW602, RW507, CW601, CW602

5.1.3 Enhancementment of antimicrobial compound synthesis by cross signalling induction

Two pure strains ofbacteria CW602 and CW401 has shown high antimicrobial activity, these two strains are taken for production of antimicrobial compounds by cross signalling induction of the pathogen against whom they have shown more antimicrobial activity. As microbial production of antibiotics is an adaptive defence mechanism,which is activated in the presence of competing organisms (Patterson and Bolis., 1997). So in our present study we also fermented the two strain in their logarthimic phase with the co cultivation of the respective pathogen *Pseudomonas* sp.,.and *Bacillus* sp. and studied the antimicrobial activity according to the methods described in the material and methods chapter.The antimicrobilal activity was measured as zone of inhibition (mm) and given in the Table-3

TABLE 3 – production of antimicrobial activity by marine bacteria exposed to heat killed as well as live cells of *Pseudomonas* and *Bacillus* sp.(inducer strains)

Marine Bacterial Strain	***Pseudomonas* sp.**			***Bacillus* sp.**		
	Control	**Heat killed cells**	**Live cells**	**Control**	**Heat killed cells**	**Live cells**
CW 602	7	13	17	NA	NA	NA
CW 402	NA	NA	NA	8	15	18

In the controls only Zobell's marine broth was added.

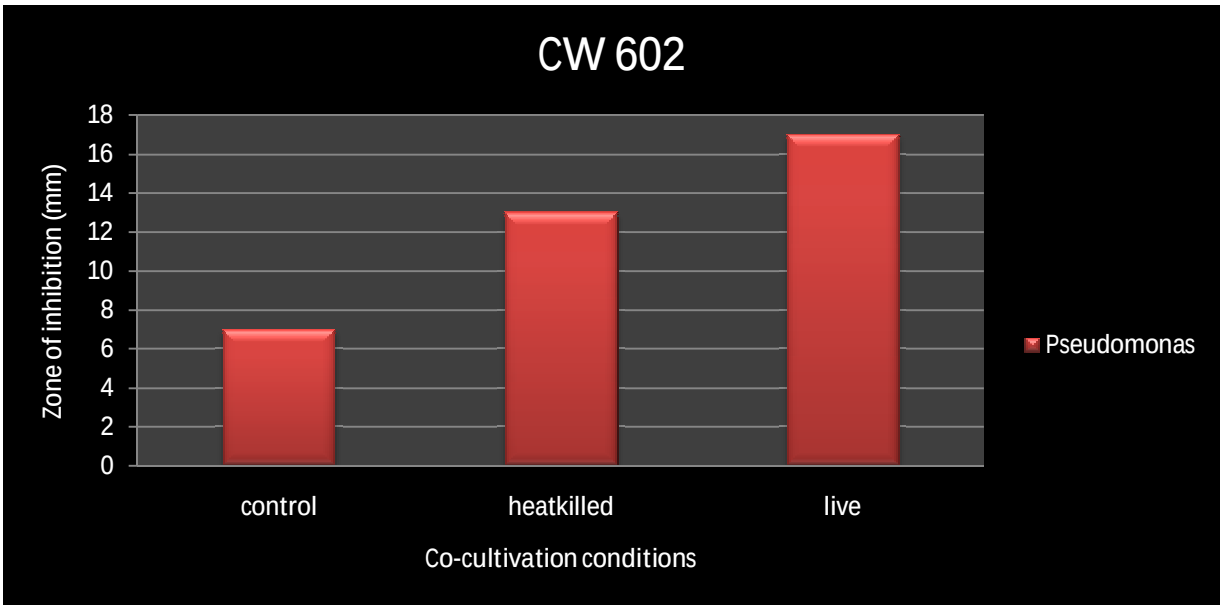

Fig. 7 : Production of antibacterial compounds by bacterial strain (CW 602) in response to live and heat killed *Pseudomonas* sp.

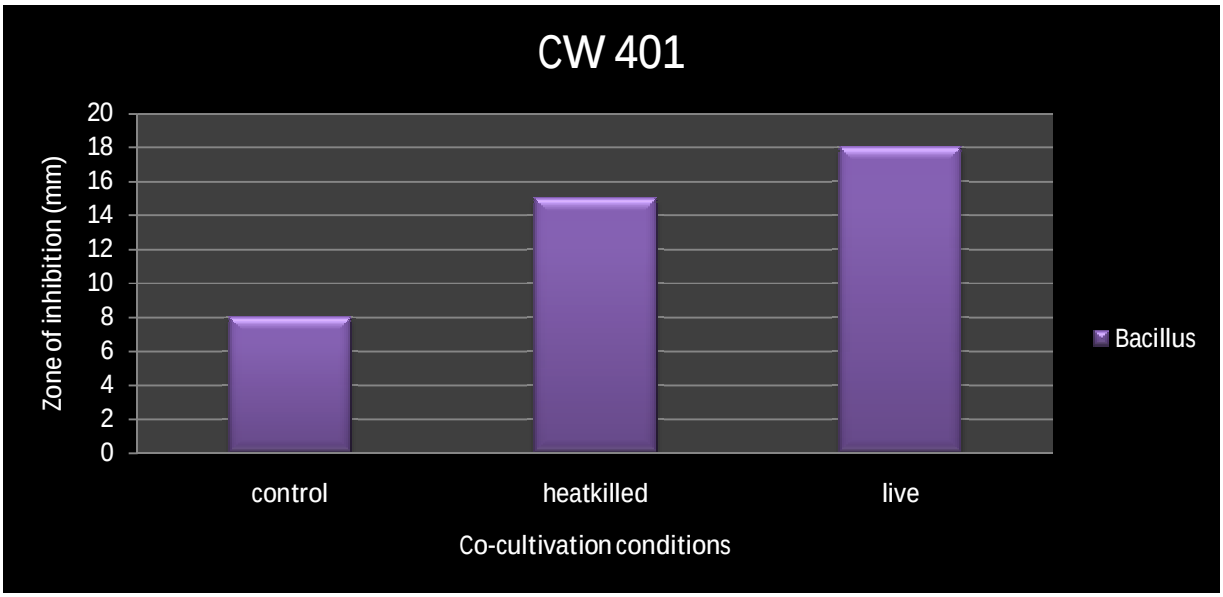

Fig. 8 : Production of antibacterial compounds by bacterial strain (CW 401) in response to live and heat killed *Bacillus* sp.

5.2 Characterization Of Bacterial Strains:-

5.2.1 Physical Characterization:

The isolated bacterial species CW401, RW507, CW601 & CW602 were morphologically characterized by Gram straining and were found to be Gram negative Staphylococcus, Gram negative Staphylococcus, Gram positive Streptococcus and Gram negative Staphylococcus.

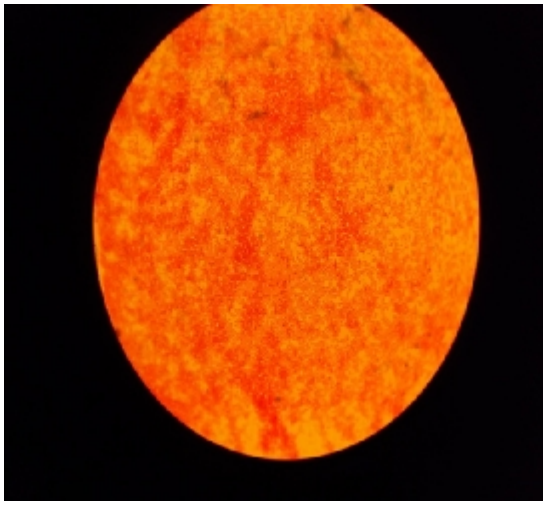

Fig. 9 : a) Microscopic view of CW401

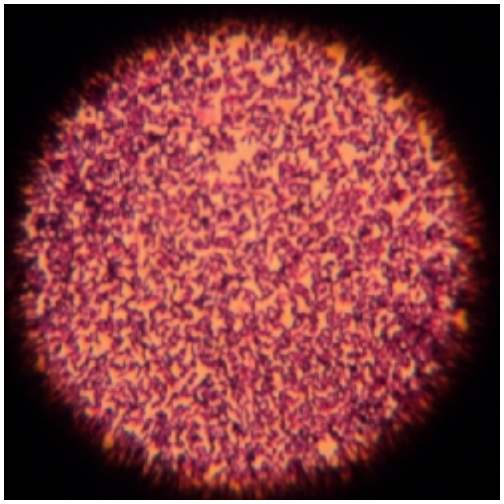

Fig 9 : b)Microscopic view of RW507

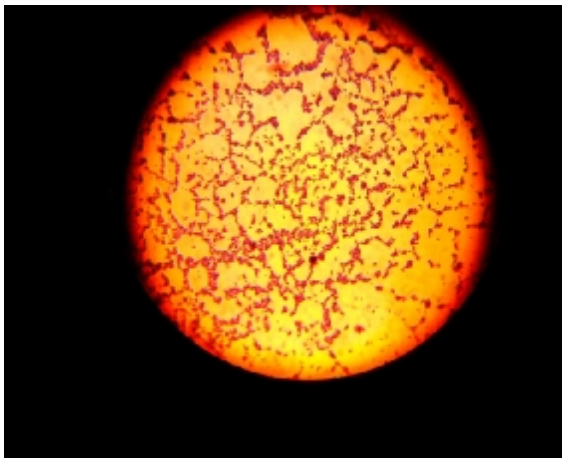

Fig.9 : c) Microscopic view of CW601

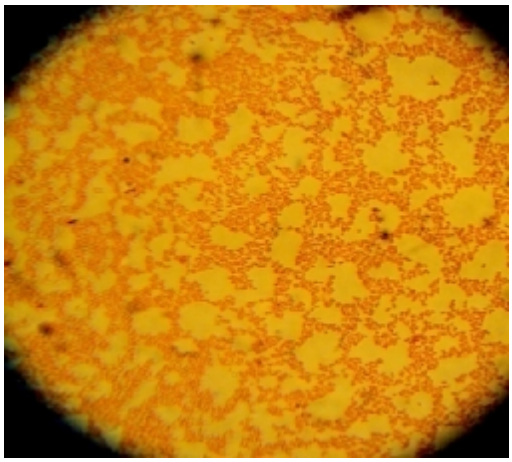

Fig 9: d) Microscopic view of CW602

TABLE 4- Physical Characterization of the strains by Gram Staining

Strains	**Colour& Shape**	**Type of bacteria**
CW401	Pink & rod shaped (*Staphylobacilli*)	Gram Negative
RW207	Pink &Rod shaped (*Staphylobacilli*)	Gram Negative
CW601	Blue & coccus (*Steptococcus*)	Gram positive
CW602	Pink & rod shaped (*staphylobacilli)*	Gram Negative

5.2.2 Biochemical tests results:-

TABLE 5- Biochemical Identification of strains using High *Bacillus* Kit

Test	CW401	RW507	CW601	CW602
Malonate	-	-	-	+
Voges proskauer	-	-	-	+
Citrate	+	-	+	+
ONPG	+	-	+	+
Nitrate Reduction	+	-	-	+
Catalase	+	-	-	+
Arginine	+	+	+	+
Sucrose	+	+	+	+
Mannitol	+	+	+	+
Glucose	+	+	+	+
Arabinose	-	-	-	+
Trehalose	+	+	+	+

Fig. 10: a) (Tests order for CW 401 & RW507 - Malonate test, Voges proskauer test, citrate test, ONPG test, Nitrate reduction, catalase test, Arginine test, Sucrose test, mannitol test, Glucose test,Arabinose test, Trehalose test)

Fig. 10: b) (**Tests order for CW 601 & CW602 -** Malonate test, Voges proskauer test, citrate test, ONPG test, Nitrate reduction, catalase test, Arginine test, Sucrose test, mannitol test, Glucose test,Arabinose test, Trehalose test)

6. DISCUSSION

It has been well established that antibiotic production can be induced or enhanced by exposing producing strains with competing organisms (Patterson and Bolis., 1997). In 1998, Spragg et al. , showed that both live and heat killed cells of *S. aureus* induced production of antibiotics in two marine strains (Mbbc 1122 and Mbbc1123). They suggested the possible rationale for the enhancement of antibiotic production in bacteria, as due to the competition for space, nutrient and to deter or kill potential competitor bacteria in the environment. As the concept of the survival of the existance the marine bacteria are also compete for the nutrients and the available space for growing for which they are induced and forced to secrete some molecules that can help them for the existance of that environment and rest to eliminate. The results of the present co-cultivation study confirm that the isolate CW 602 and CW 401 produced antimicrobial compounds more amounts in the presence of *Pseudomonas* sp. and *Bacillus* sp. against whom they are reactive. Similar observations have been made by Spragg et al., (1998), in which surface-associated marine bacteria AMS1·6 produced antibacterial compound only in response to the presence of methicillin susceptible/ resistant *S. aureus.*

Results of the present co-cultivation study depict interspecies quorum-sensing pattern where *Pseudomonas* sp.. induced antimibacterial compound production in CW 602 and and *Bacillus* sp. induced in CW 401. There are few reports available regarding interspecies relationship between Bacteria with other bacterial organisms in response to antibacterial compound production. Robles and Joanne (2006) reported that out of 53 bacterial isolates obtained from soil, 33 isolates including *Bacillus* sp , *Micrococcus*, *Stenotrophomonas* and *Lysobacter* were found to induce antibiotic production in S. coelicolor through molecular signals. Similarly in 2001, Slattery et al., examined the impact of co-culture of marine bacteria on istamycin antibiotic production by *S. Tenjimariensis* with 53 different bacterial species and found that 12 bacterial strains induced the production of istamycin and this antibiotic inhibited growth of other competitor bacterial colonies.

In the present study, a strain of *Pseudomonas* sp. was introduced into CW 602 and *Bacillus* sp. was introduced into CW 401 culture during growth phase by adopting the method described by

Kanagasabhapathy and Nagata (2007), in contrast to the method of Spragg et al., (1998). Mixing of live/ heat killed *Pseudomonas* sp. and *Bacillus* sp. with Bacteria will facilitate the transfer of signal molecules produced by the inducer. Kanagasabhapathy and Nagata (2007) observed that, *B. licheniformis* when grown as pure culture did not produce any activity against marine fouling bacteria but, when challenged with fouling bacteria (FB-9), it produced antibacterial compound. In the present study, strain CW 602 and CW 401 when cultured alone in a shake flask produced very less amount of antibacterial compound(s). The isolate CW 602 and CW 401 produced antibacterial compound(s), only when co cultured with live and /or heat-killed cells of *Pseudomonas* sp. and *Bacillus* sp.(figure 7 and 8).

7. CONCLUSION

- Antibiotic production can be induced or enhanced by exposing producing strains with competing organisms
- The results of the present co-cultivation study confirm that the isolate CW 602 and CW 401 produced antimicrobial compounds in more amounts in the presence of Pseudomonas sp. and Bacillus sp. against whom they are reactive.
- Results of the present co-cultivation study depict interspecies quorum-sensing pattern where Pseudomonas sp.. induced antimibacterial compound production in CW 602 and and Bacillus sp. induced in CW 401.
- Mixing of live/ heat killed Pseudomonas sp. and Bacillus sp. with Bacteria will facilitate the transfer of signal molecules produced by the inducer.
- In the present study, strain CW 602 and CW 401 when cultured alone in a shake flask produced very less amount of antibacterial compound(s). The isolate CW 602 and CW 401 produced antibacterial compound(s), only when co cultured with live and /or heat-killed cells of Pseudomonas sp. and Bacillus sp

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